

Factor XI Regulates Pathological Thrombus Formation on Acutely Ruptured Atherosclerotic Plaques

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Factor XI Regulates Pathological Thrombus Formation on Acutely Ruptured Atherosclerotic Plaques

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Objective—Coagulation factor XI is proposed as therapeutic target for anticoagulation. However, it is still unclear whether the antithrombotic properties of factor XI inhibitors influence atherosclerotic disease and atherothrombosis. Our aim is to investigate whether factor XI antisense oligonucleotides could prevent thrombus formation on acutely ruptured atherosclerotic plaques.

Approach and Results—Atherosclerotic plaques in the carotid arteries of *Apoe*^{-/-} mice were acutely ruptured using ultrasound. The subsequent thrombus formation was visualized and quantified by intravital microscopy and immunohistochemistry. Mice were pretreated with either factor XI antisense or nonsense oligonucleotides (50 mg/kg) to lower factor XI plasma levels. A tail bleeding assay was used to determine the safety. On plaque rupture, initial platelet adhesion and platelet plug formation were not impaired in animals treated with factor XI antisense oligonucleotides. However, the ensuing thrombus formation and fibrin deposition were significantly lower after 5 to 10 minutes ($P < 0.05$) in factor XI antisense oligonucleotide-treated animals without inducing a bleeding tendency. Furthermore, thrombi from antisense-treated animals were less stable than thrombi from placebo-treated animals. Moreover, macrophage infiltration and collagen deposition were lower in the carotid arteries of factor XI antisense-treated animals. No neutrophils were present.

Conclusions—Factor XI antisense oligonucleotides safely prevent thrombus formation on acutely ruptured atherosclerotic plaques in mice. Furthermore, perturbed carotid arteries from factor XI antisense-treated animals show a less severe inflammatory response. (*Arterioscler Thromb Vasc Biol.* 2014;34:1668-1673.)

Key Words: atherosclerosis ■ blood coagulation ■ factor XI ■ thrombosis

Atherosclerosis is the underlying cause of $\approx 50\%$ of all deaths in developed countries.¹ An important reason for this chronic inflammatory disorder to become life threatening is thrombus formation on ruptured atherosclerotic plaques, resulting in myocardial infarction, peripheral artery disease, and stroke. Primary treatment of these disorders is with antiplatelet (eg, aspirin and clopidogrel) and anticoagulant drugs (eg, warfarin, heparins, factor Xa, and thrombin inhibitors).² These drugs are undeniably good in terms of effectiveness but have serious side effects of which bleeding is the most pronounced.³ To overcome these bleeding problems, coagulation factor XI has been proposed as alternative target for anticoagulation⁴⁻⁷ because factor XI is associated with thrombosis, whereas it has a relatively minor role during normal hemostasis. This means that normal clot formation is still preserved in the absence of factor XI, while thrombosis is prevented. For example, inhibition of factor XI protects rodents and primates from experimentally induced thrombosis in both arteries and veins.⁸⁻¹²

Another argument for factor XI as antithrombotic target is the epidemiological evidence that patients with factor XI deficiency have a lower incidence of deep vein thrombosis and ischemic stroke.^{13,14}

See accompanying editorial on page 1607

Until now, little is known about the role of factor XI inhibition in atherosclerosis and subsequent atherothrombosis. This is an important extension because atherosclerotic plaques contain many components and cells with a thrombogenic potential beyond the coagulation cascade, such as oxidized low-density lipoproteins, lysophosphatidic acid, and macrophages.^{15,16} In addition, tissue factor (TF) and collagen are important determinants of thrombus formation on ruptured plaques.¹⁵ The question is whether factor XI inhibition is capable to overcome this prothrombotic environment. Because coagulation is not dependent on factor XI when high concentrations of TF are present,^{17,18} we questioned whether factor XI inhibition was still effective to prevent atherothrombosis.

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Nonstandard Abbreviation and Acronym	
TF	tissue factor

Furthermore, atherothrombotic disorders, such as myocardial infarction and stroke, are diseases of the elderly and occur in patients with pronounced atherosclerotic vessels. In contrast, many animal experiments have been performed in young (4–8 weeks) mice with healthy nonatherosclerotic vessels. In this study, we investigated the antithrombotic potential of factor XI antisense oligonucleotides in a model of plaque rupture, which is sensitive to both antiplatelet and antithrombotic therapy,¹⁵ in older (18–20 weeks) mice with pronounced atherosclerotic vessels.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Factor XI Affects Thrombus Formation on Ruptured Plaques

The role of factor XI in mouse models has only been established in artificially damaged healthy vessels. Here, we studied the role of factor XI in thrombus formation after acute rupture of an atherosclerotic plaque in diseased vessels. Factor XI reduction was accomplished by injection of factor XI antisense oligonucleotides (50 mg/kg) in *Apoe*^{-/-} mice fed with a high-fat diet to produce sufficient atherosclerosis. Three injections with factor XI antisense oligonucleotides significantly reduced plasma factor XI activity levels to 20% (Figure 1A). Because the factor XI levels in the control group showed some variation, we normalized these levels for prothrombin. As depicted in Figure 1B, prothrombin levels were not significantly different between groups. Subsequently, we calculated the mean factor XI/prothrombin ratio for both controls (0.36) and factor XI antisense (0.13)-treated animals. By dividing these ratios, we found that factor XI levels were 2.8× lower in the plasma of factor XI antisense-treated animals than in the control group, corresponding to a 64% reduction of factor XI plasma levels.

Initially, in vivo thrombus formation after plaque rupture occurred similarly in both control *Apoe*^{-/-} mice and *Apoe*^{-/-} mice pretreated with factor XI antisense oligonucleotides (Figure 2A and 2B; Movies I and II in the online-only Data

Supplement). However, pretreatment with factor XI antisense oligonucleotides reduced thrombus propagation and fibrin deposition, an effect that became significant 5 minutes after plaque rupture and continued for 10 minutes (Figure 2A and 2B). Furthermore, the platelet aggregates seemed to be less stable than the thrombi formed in animals treated with non-sense oligonucleotides as depicted by the number of platelets shed from the initial thrombus (Figure 2C). This indicates that inhibition of factor XI results in less stable clots, which should be a local event because systemic D-dimer levels were not different between groups (data not shown). Treatment with factor XI antisense oligonucleotides is safe in mice because the bleeding time was unaffected by the oligonucleotides (Figure 2D).

Reduced Thrombus Formation Is Partly Because of a Less Severe Inflammatory Response

To study the thrombi and plaques after the rupturing procedure in more detail, we poststained the carotid arteries and subjected these to immunohistological analysis. Thrombus phenotype characteristics were determined by staining the sections for fibrin, macrophage differentiation antigen-3 (macrophage infiltration), lymphocyte antigen-6G (neutrophil recruitment), and with Sirius red (collagen) and hematoxylin and eosin. In the arteries of control *Apoe*^{-/-} mice, thrombi contained many erythrocytes as a major component of a thrombus (Figure 3A). Factor XI antisense oligonucleotides significantly reduced thrombus size ($14.1 \pm 4.0 \times 10^3 \mu\text{m}^2$ total thrombus size) when compared with placebo-treated animals ($68.4 \pm 23.9 \times 10^3 \mu\text{m}^2$; $n=4$ per group; $P<0.05$) as shown in Figure 3B. Furthermore, using an antibody against fibrinogen, we observed fibrin fibers in the same thrombus within the carotid artery (Figure 3C, in brown; $29.9 \pm 10.2\%$ of thrombus area; Figure 3D). In contrast, hardly any thrombi were observed in the carotid arteries of animals treated with factor XI antisense oligonucleotides as shown in Figure 3A and 3C ($6.4 \pm 2.6\%$ of thrombus area; Figure 3D; $n=4$ per group; $P<0.05$). Finally, to confirm that the thrombi are formed on a ruptured atherosclerotic plaque, we visualized the fibrous cap of the plaque using a Sirius red staining (Figure 3E). The fibrous cap with subsequent thrombus formation was only visible in control animals and not in the factor XI antisense-treated animals.

The (innate) immune system contributes to the initiation and amplification of venous thrombosis in mice.¹⁹ Especially, monocytes and neutrophils seem to be responsible for

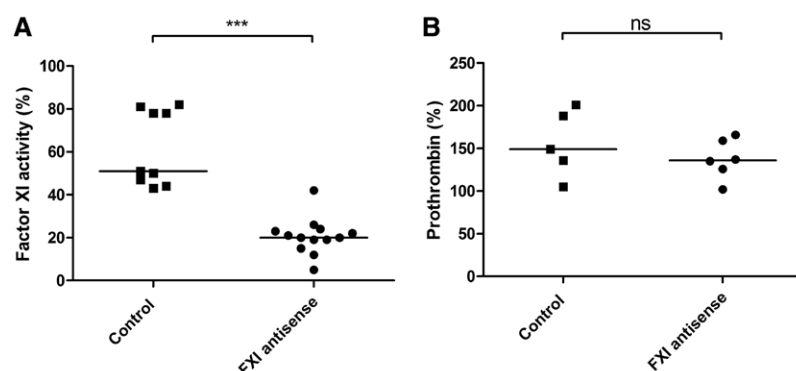


Figure 1. A and B, Effect of factor XI (FXI) antisense on FXI activity and prothrombin levels in mouse plasma. Male *Apoe*^{-/-} mice were fed a high-fat diet for 18 to 20 weeks, after which they were treated subcutaneously every 3 to 4 days with FXI antisense oligonucleotides for 10 days (dose 50 mg/kg). Each symbol represents 1 animal, and the horizontal line indicates median, *** $P<0.0001$, $n=9$ for the control group and $n=13$ for the FXI antisense-treated group. ns indicates nonsignificant.

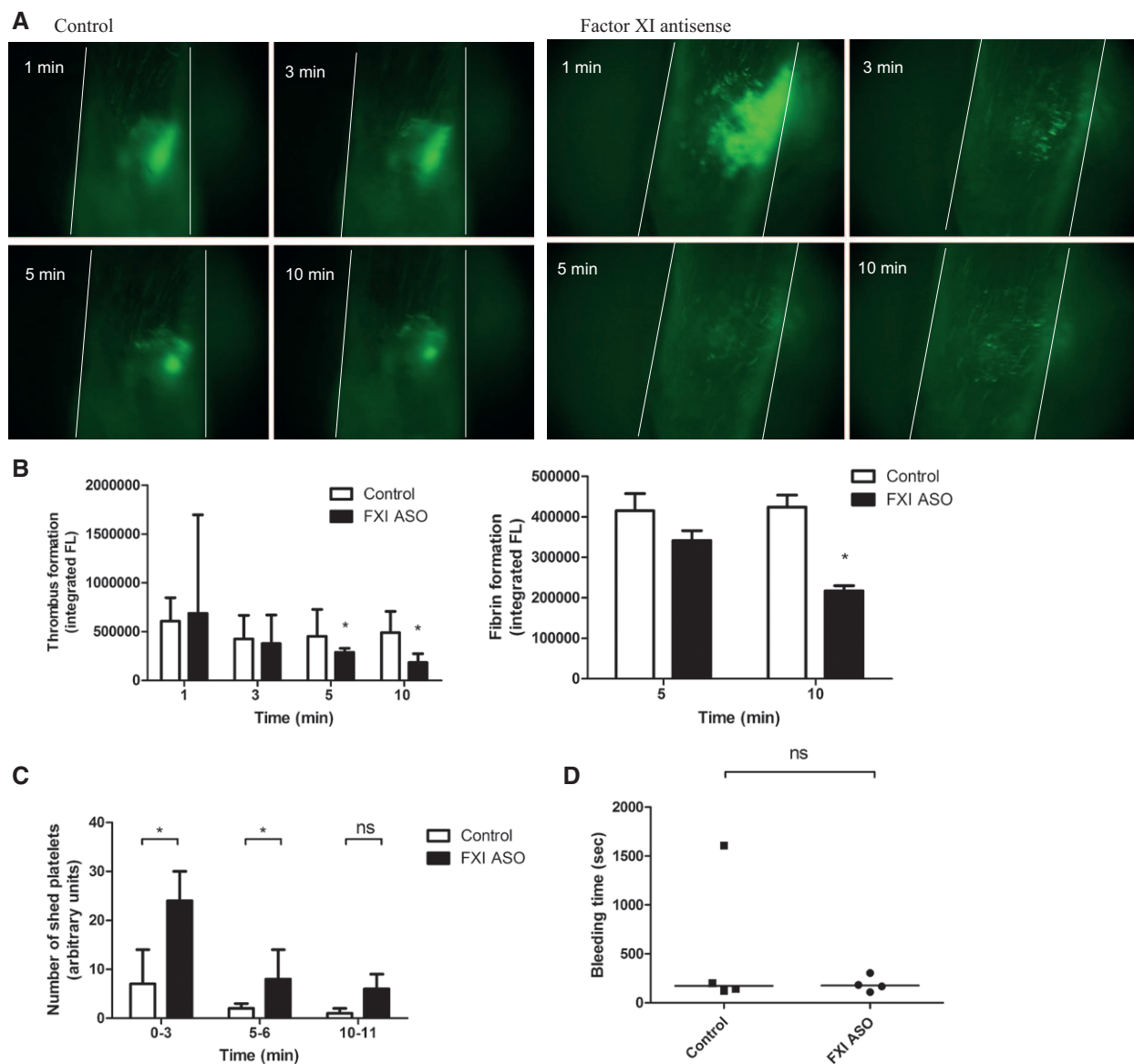


Figure 2. Effect of factor XI (FXI) antisense on thrombus formation on acutely ruptured atherosclerotic plaques. The carotid artery of *Apoe*^{-/-} mice fed on a Western-type diet for 18 to 20 weeks was dissected free from surrounding tissue. Visible plaques were ruptured using an ultrasound wave. Subsequent thrombus formation was registered using intravital microscopy for a maximum of 30 minutes. Mice were treated with either FXI antisense (50 mg/kg; 3 injections) or nonsense oligonucleotides (control; 50 mg/kg; 3 injections). Depicted are representative fluorescent images after 1, 3, 5, and 10 minutes (**A**) and a quantification of thrombus formation and fibrin deposition using the integrated fluorescence intensity as described in the Materials and Methods in the online-only Data Supplement (**B**). Each bar graph represents the median value of 9 animals, error bars indicate interquartile ranges (IQR), **P*<0.05. The number of fluorescent platelets shed were counted (independently by 2 investigators) for the first 3 minutes after plaque rupture and for 1 minute after *t*=5 and *t*=10 (**C**). Data are medians with IQR, **P*<0.05. Atherosclerotic mice were given 3 doses of either factor XI antisense (50 mg/kg) or nonsense oligonucleotides (50 mg/kg). Tail bleeding was evaluated as described in the Materials and Methods in the online-only Data Supplement (**D**). Each symbol represents 1 animal (*n*=4 per group), and the horizontal line indicates median. ns indicates nonsignificant.

thrombus formation. Therefore, we stained the carotid arteries for the presence of macrophages and neutrophils to investigate whether these cells are also involved during thrombus formation on ruptured atherosclerotic plaques. As shown in Figure 3F and 3G, there was a higher degree of macrophage infiltration in thrombi from placebo-treated animals than in factor XI antisense-treated animals ($18.5 \pm 1.1\%$ versus 11.4 ± 2.6 of thrombus area; *n*=4 per group; *P*=0.05). However, we did not observe any neutrophil infiltration in the thrombi from both control animals and factor XI antisense-treated animals (Figure 3H).

Discussion

The results of our study demonstrate that initial clot formation on atherosclerotic plaques is not impaired in mice treated with factor XI antisense drugs. Platelet adhesion and aggregation were not significantly different between treatment groups, which imply that primary hemostasis is not influenced by factor XI inhibition. However, because clots were significantly smaller after 5 to 10 minutes in the factor XI antisense group when compared with controls, this shows that secondary hemostasis is impaired by factor XI inhibition.

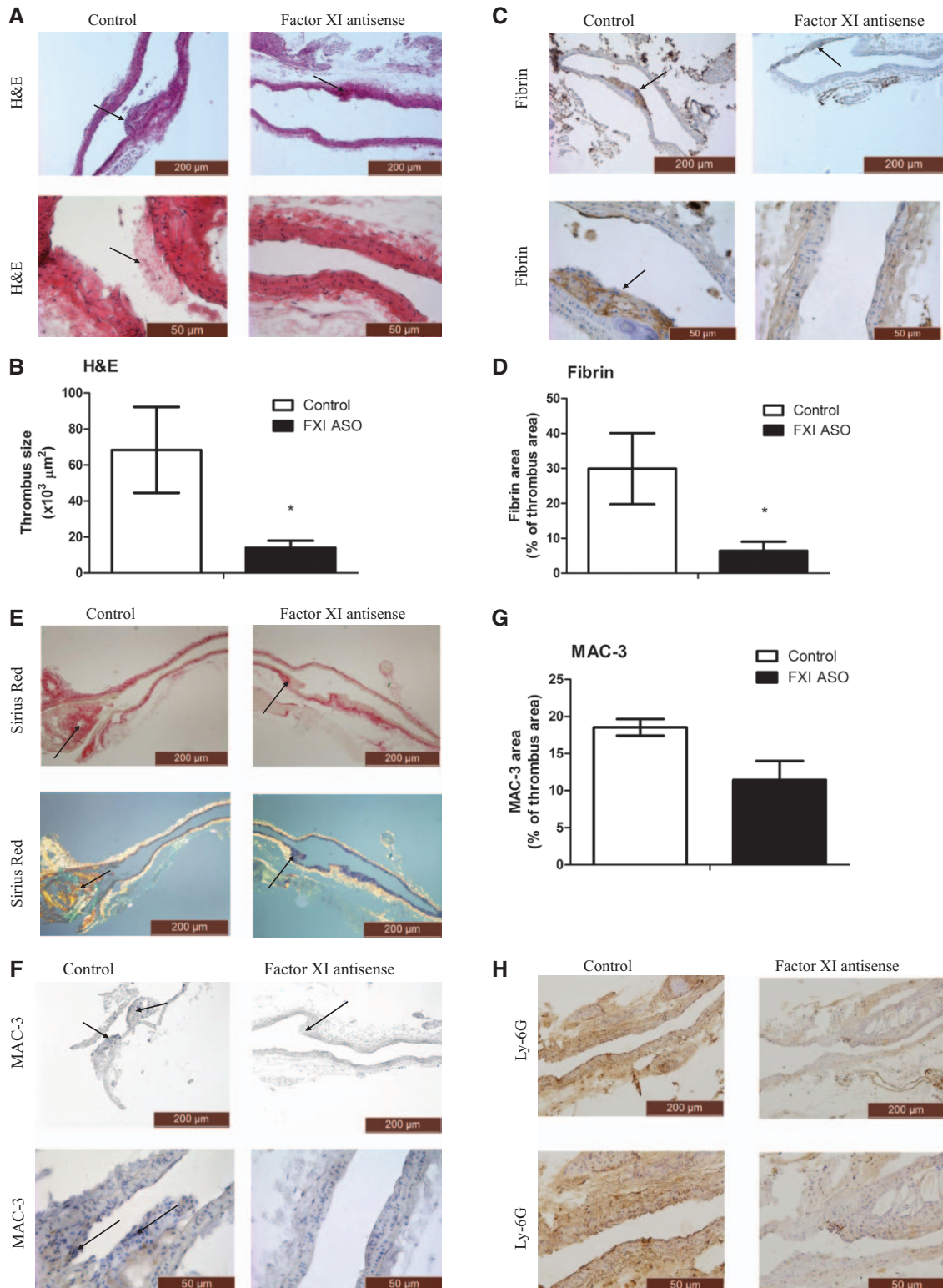


Figure 3. The role of factor XI in coagulation and inflammation. **A**, Images of the carotid artery stained with hematoxylin and eosin, used to visualize the extent of atherosclerotic plaque burden and erythrocyte infiltration. The arrow indicates a ruptured plaque. **B**, There was a significant difference in thrombus size between factor XI (FXI) antisense and placebo-treated animals. **C**, Sections were stained with an antifibrin(ogen) antibody to study thrombus formation. The arrow indicates the formed thrombus, with the fibrin threads in brown. **D**, Fibrin deposition was significantly lower in thrombi from factor XI antisense-treated animals when compared with placebo. **E**, The fibrous cap was visualized with a collagen staining (Sirius red) both in bright-field (top) and polarized light (bottom), the arrow indicates positive staining. **F**, Macrophage infiltration at the ruptured site was analyzed using macrophage differentiation antigen-3 staining. The arrow indicates positive staining; macrophages are in brown. **G**, Macrophage infiltration was not significantly different between the 2 treatment groups. **H**, Finally, the sections were stained for neutrophils using a lymphocyte antigen-6G antibody; however, no positive staining was observed. Error bars represent mean \pm SEM; * P <0.05. Representative lower (bar, 200 μ m) and higher (bar, 50 μ m) magnification images are given.

This is the first study that focused on factor XI inhibition in diseased atherosclerotic vessels of older animals. Because atherosclerotic plaques contain a variety of prothrombotic substances, including TF, it is important to study the process of clot formation in ruptured plaques. We know from *in vitro* and *in vivo* studies that thrombus formation is probably less dependent on factor XI when there is a high concentration of TF.^{17,18} Furthermore, several other thrombogenic substances, such as oxidized low-density lipoproteins and macrophages, are exposed to the bloodstream after plaque rupture.¹⁵ Despite these prothrombotic conditions, factor XI inhibition resulted in smaller thrombi, which suggests that factor XI antisense may be used in patients having atherosclerosis-related disorders, such as myocardial infarction and stroke. Because the plaque rupture model is known to be dependent on TF, these data indicate that, at least in mice, TF is a limiting factor in the *in vivo* coagulant activity, making the whole system dependent on factor XI. This observation on factor XI provides excellent possibilities for the secondary prevention of cardiovascular patients. Furthermore, previous experiments with this model have shown that this model relies on collagen exposure and that thrombus formation is inhibited by glycoprotein VI inhibition and ADP receptor blockage, as well as inhibition of thrombin.^{15,20} Hence, we are able to conclude that thrombus formation on these ruptured plaques relies on both platelet activation (via glycoprotein VI and P2Y12) and coagulation.

We observed no differences in bleeding time between factor XI antisense and nonsense oligonucleotide-treated animals. This does not imply that (complete) factor XI inhibition in humans is safe. Patients with a severe factor XI deficiency may have a bleeding diathesis, whereas factor XI knockout mice do not have an increased bleeding tendency. This indicates that there is a discrepancy between mice and humans about factor XI levels and bleeding.²¹

Because of the half-life of factor XI (≈ 48 hours), it takes 2 to 3 days before an antithrombotic effect is observed in mice and men. In humans, because of multiple factors, it will probably take 1 to 3 weeks before antithrombotic protection can be achieved. These factors include the time needed for the factor XI antisense oligonucleotides to distribute effectively to hepatocytes and clearance of the existing factor XI in circulation, which is dependent on its half-life. Nevertheless, this is a potential disadvantage of the drug. However, a treatment strategy incorporating heparins to bridge these first weeks before factor XI levels are reduced is probably a good solution. Most likely, the primary therapeutic use of factor XI antisense oligonucleotides will be for prevention and treatment of thrombosis in conditions, such as atrial fibrillation and venous thromboembolism. Obviously, this has to be established in clinical trials.

Interestingly, clots were not only smaller but also more unstable in factor XI antisense-treated animals. Because we observed limited amounts of fibrin, this would suggest that the instability is because of insufficient thrombin generation and fibrin formation, rather than to the disintegration of preformed rigid fibrin clots. Reduced thrombus stability in factor XI-deficient mice has been described before²²; however, the explanation for this is not entirely clear. One possibility is that

there is reduced activation of thrombin activatable fibrinolysis inhibitor, which protects the clot from degradation by plasmin. This might cause enhanced fibrinolysis with less stable clots. An argument against this theory is that D-dimer levels were not significantly different between factor XI antisense- and nonsense-treated animals.

Because mice with an atherosclerotic phenotype do not develop spontaneous thrombosis, an artificial trigger is necessary.²³ Common methods, such as ferric chloride or Rose Bengal, produce large thrombi, which fully occlude the lumen of the vessel.²⁴ However, thrombi formed on ruptured plaques in humans hardly ever cause full occlusion of the vessel.^{25,26} In addition, these methods do not expose the content of the plaque core with all its thrombogenic substances to the bloodstream. In our model, plaque rupture was induced with an ultrasound wave, which induced thrombus formation without affecting blood flow.¹⁵ The formed thrombi never fully occluded the vessel but did expose the plaque core to the bloodstream. Therefore, we think that this is an excellent model to study the dynamic process of thrombus formation on ruptured plaques. However, it is still a mouse model with an artificial trigger, so our model has to be translated to humans with some caution.

Inhibition of factor XI activation attenuates inflammation,²⁷ and indeed we found that macrophage infiltration was lower in thrombi from factor XI antisense-treated animals. This was only observed in thrombi formed on the ruptured plaques and not in the underlying plaque itself. Because factor XI activity might be involved in pathways that affect leukocyte function and trafficking, this observation could merely reflect a decrease in circulating leukocytes.²⁸ At present, there is no known mechanistic link between factor XI activation and leukocyte function although there is evidence that factor XIa has direct cellular signaling functions through various receptors.²⁸ Furthermore, factor XI is, together with factor XII and prekallikrein, a part of the contact system that constitutes a link between coagulation and inflammation,²⁹ and inflammatory cells, such as activated neutrophils, are proposed to trigger coagulation.¹⁹ Therefore, inhibiting factor XI might suppress inflammatory pathways, which are responsible for pathological thrombus formation on atherosclerotic plaques.

We conclude that inhibition of factor XI prevents thrombosis on atherosclerotic plaques. The question remains whether factor XI inhibition influences the process of atherogenesis. Several clotting factors, including thrombin, contribute to atherosclerotic plaque formation.³⁰ Therefore, it would be interesting to study the role of factor XI during this long-term process. Our current observations are that inhibition of factor XI prevents thrombosis on acutely ruptured atherosclerotic plaques without impairment of normal clot formation and indicates that targeting factor XI could be a safe alternative for the prevention of atherothrombotic disease.

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Disclosures

S Bhanot and B.P. Monia are employees of ISIS Pharmaceuticals. The other authors report no conflicts.

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Significance

This is the first study that establishes the importance of coagulation factor XI during atherothrombotic diseases. Current opinion assumes that atherothrombotic disorders, such as myocardial infarction and stroke, are mainly a platelet-driven event, accompanied by tissue factor–initiated coagulation. In this study, we show that thrombus formation on acutely ruptured atherosclerotic plaques is dependent on factor XI, and that the thrombotic burden can be decreased by reducing factor XI levels using factor XI antisense oligonucleotides. Furthermore, inhibition of factor XI is not associated with an increased bleeding tendency, which indicates that targeting factor XI could be a safe alternative for the treatment of atherothrombotic disease.